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**Comparative thyroidology: thyroid gland location and
iodothyronine dynamics in Mozambique tilapia (*Oreochromis
mossambicus* Peters) and common carp (*Cyprinus carpio* L.).**

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Summary

In teleosts, the thyroid gland is mostly found in the subpharyngeal area. However, in several species thyroid follicles are found in, e.g., heart, head kidney and kidney. Such heterotopic thyroid follicles are active, and considered to work in concert with the subpharyngeal thyroid. In Mozambique tilapia (*Oreochromis mossambicus*) thyroid activity is, indeed, restricted to the subpharyngeal area; in common carp (*Cyprinus carpio*) the functional endocrine thyroid is associated with renal tissues. The subpharyngeal follicles of carp comprise only 10% of the total thyroid tissue, and these follicles neither accumulate iodide nor synthesise or secrete thyroid hormones to a significant degree. Although shape and size of carp subpharyngeal and renal follicles vary, the epithelial cell height of the thyrocytes and thyroxine immunoreactivity do not differ, which suggests that the activity of the carp subpharyngeal thyroid follicles is dormant. Differences in thyroid physiology between both fish species were further assessed at the level of peripheral thyroid hormone metabolism. Carp clears plasma faster of thyroid hormones than tilapia. Furthermore, a significant amount of conjugated thyroid hormones was observed in the plasma of tilapia, which was preceded by the occurrence of thyroid hormone conjugates in the subpharyngeal area and the appearance of conjugates in the surrounding water. Apparently, plasma thyroid hormone conjugates in tilapia originate from the thyroid gland and function in the excretion of thyroid hormones. Our data illustrate the variability in teleostean thyroidology, an important notion for those studying thyroid physiology in fishes.

Introduction

The main products of the thyroid gland are thyroid hormones which actions are pleiotropic and involve the regulation of *i.a.* metabolism, growth and development, including metamorphosis.

Thyroid hormones are synthesised in thyroid follicles, the functional units of the thyroid gland

40 composed of thyrocytes enclosing a protein filled matrix, the colloid. Thyroid stimulating hormone (TSH) from the pituitary gland is the major stimulus for thyroid hormone synthesis and release (Blanton and Specker, 2007; Eales and Brown, 1993). Plasma thyroid hormone levels are not only determined by thyroid hormone synthesis and secretion, but also by peripheral metabolism (*viz.* deiodination and conjugation), clearance and excretion of thyroid
45 hormones. Thyroid hormones are generally excreted as glucuronide or sulphate conjugates via the bile (Finnsen and Eales, 1996). Unlike the compact mammalian thyroid gland, the thyroid gland of most teleostean fishes consists of non-encapsulated follicles scattered in the subpharyngeal area surrounding the ventral aorta (Gudernatsch, 1911). In several species of fish, however, heterotopic thyroid follicles, *i.e.* follicles located outside the typical subpharyngeal area
50 have been reported (Baker, 1958).

Heterotopic thyroid follicles can be found near or in the heart, spleen, liver, oesophagus, brain and choroid rete mirabile of fish, but are generally restricted to tissues that ontogenetically derive from renal primordia, *viz.* the head kidney (pronephros) and the adult kidney (opisthonephros) (Baker, 1958). Thyroid heterotopia has been described in species throughout
55 the Teleostei infraclass; it is found in representatives of the order of anchovies and herrings (Clupeiformes, 1 species), catfish (Siluriformes, 4 species), killifish (Cyprinodontiformes, 3 species), swamp eels (Synbranchiformes, 1 species), perch-like fishes (Perciformes, 3 species), rainbow fish and silversides (Atheriniformes, 1 species) and minnows and suckers (Cypriniformes, 14 species). Interestingly, 13 of the 27 fish species in which heterotopic thyroid
60 follicles are described belong to the family of carp and minnows (Cyprinidae), including species such as goldfish (*Carassius auratus*) and common carp.

Because of their ectopic nature, heterotopic thyroid follicles have often been misinterpreted as to result from metastases (Berg et al., 1953; Blasiola et al., 1981; Nigrelli, 1952). Although thyroid hyperplasia and neoplasia have been described in teleostean fishes (Fournie et al., 2005; Leatherland and Down, 2001), normal heterotopic thyroid follicles do not follow the diagnostic criteria for thyroid hyperplasia, adenoma or carcinoma as proposed by Fournie et al. (2005).

Most reports on heterotopic thyroid follicles in fish only describe the presence of heterotopic thyroid follicles without consideration as to quantitative or functional aspects (Agrawala and Dixit, 1979; Baker, 1958; Qureshi, 1975; Qureshi et al., 1978; Qureshi and Sultan, 1976; Sathyanesan, 1963). Extra-pharyngeal thyroid populations have been reported to be less (Bhattacharya et al., 1976), equal (Frisén and Frisé, 1967) or more active (Chavin and Bouwman, 1965; Peter, 1970; Srivastava and Sathyanesan, 1971) than the subpharyngeal thyroid tissue. The general opinion is that these heterotopic follicles work in concert with the subpharyngeal thyroid and contribute partly to the thyroid status of an animal.

Since iodide is exclusively incorporated into thyroid hormones and its metabolites, the use of radioactive isotopes of iodide offers unique possibilities for the investigation of thyroid hormone dynamics. A serendipitous observation during autoradiography of the thyroid gland in Mozambique tilapia (*Oreochromis mossambicus* Peters) and common carp (*Cyprinus carpio* L.) revealed differences in iodide metabolism, and pointed to the presence, in carp, of heterotopic thyroid tissue that is functionally different from that in the subpharyngeal region. This was the motivation for the studies described here.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) of the all-male E4×R3R8 isogenic strain (Bongers et al., 1998) were obtained from the Department of Fish Culture and Fisheries of Wageningen University,

the Netherlands. Mozambique tilapia (*Oreochromis mossambicus* Peters) were obtained from laboratory stock. Fish were kept in 150-L tanks with aerated, recirculating city of Nijmegen tap water, at a photoperiod of 16 h light and 8 h darkness at 23°C for carp and 27°C for tilapia. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) at a daily ration of 1.5% of their estimated body weight. Animal handling followed approved university guidelines.

Whole body autoradiography

Carp and tilapia were exposed for 16.5 h to 250 $\mu\text{Ci Na}^{125}\text{I}$, which was added to the aerated water in a 3-L tank, at 23 and 27°C, respectively. The thyrostatic potassium-perchlorate (KClO_4) was added at a concentration of 1 mM, and its effect on ^{125}I uptake was compared to that in an untreated group. After exposure, fish were deeply anaesthetised with 0.1% (v/v) 2-phenoxyethanol and killed by immersion in melting isoflurane (-70°C). Animals were embedded in 5% carboxymethyl cellulose, stored at -20°C and whole-body cryosections were obtained according to a method described by Rijntjes et al. (1979). In short: a carboxymethyl cellulose block containing a specimen was mounted on the stage of a LKB 2250-PMV cryomicrotome (LKB, Stockholm, Sweden). Sections were collected with the aid of cellulose tape that was applied to the upper surface of the carboxymethyl cellulose block, and were freeze dried in the microtome for 24 h. Thirty- μm thick sections were sectioned every 90 μm . Freeze-dried whole-body sections of the whole fish were placed on Biomax MR-1 X-ray film (Eastman Kodak Company, Rochester, NY, USA), films were exposed for 3 days at -70°C after which they were developed according to the manufacturer's protocol.

Injection procedure and sampling

Carp (102 ± 14 g; $n = 24$) and tilapia (117 ± 17 g; $n = 24$) were injected intraperitoneally (i.p.) with 20.3 μCi carrier-free Na^{125}I (Amersham Biosciences, Buckinghamshire, UK) per 100 g body weight. The ^{125}I specific activity was $82 \cdot 10^{15}$ Bq/mol and the radiotracer was dissolved in 0.9%

NaCl. After injection, fish were immediately transferred to individual tanks with 3.5 L aerated
city of Nijmegen tap water. During the experiment, water was sampled and radioactivity was
measured. Fish were sampled at set times after injection by adding 0.1% (v/v) 2-
phenoxyethanol in the individual tank to induce anaesthesia. Blood was sampled by puncture of
the caudal vessels with a heparinised syringe fitted with a 23-G needle and plasma was collected
after centrifugation at 4°C (4000 g, 15 min). The fish were then killed by spinal transection and
selected organs and tissues, as indicated in the legends of the figures, were collected. All tissues
and the remaining carcass were weighed and the volume and weight of total bile and the
collected plasma sample were determined. The radioactivities of bile and plasma were measured
in an LKB 1272 Clinigamma γ -counter (Wallac, Turku, Finland) and immediately thereafter
subjected to Sephadex LH-20 chromatography (see below). All tissues were homogenized
according to Chopra et al. (1982) with an all-glass Potter-Elvehjem homogenisation device in
ice-cold 0.1 M Tris-HCl buffer (pH 8.7) added at 6 ml per gram tissue. Total radioactivity of the
homogenates was determined as described for bile and plasma. The carcass was microwaved for
3 min at 800 W and homogenised in a blender in 200 ml 0.1 M Tris-HCl buffer (pH 8.7). The
resulting total volume was assessed and the radioactivity of sextuplicate 1 ml subsamples was
determined

Histochemistry

The subpharyngeal area, head kidney and kidney of 4 carp and tilapia (39.3 ± 0.5 g) were fixed in
Bouin's solution for 24 h. Tissues were dehydrated in a graded series of ethanol, embedded in
paraplast and sectioned at 7 μ m. Every 140 μ m two serial sections were collected and mounted
on glass slides. Sections were stained with a modified Crossmon's connective tissue stain
(Crossmon, 1937): 1.3 g/L Light Green SF yellowish (Chroma-Gesellschaft, Stuttgart, Germany),
1.7 g/L Orange G (Searle Diagnostic, High Wycombe, England) and 2 g/L acid fuchsin
(Fuchsin S from Chroma-Gesellschaft, Stuttgart, Germany) were dissolved in distilled water at

80°C. The solution was cooled to room temperature, and to 50 ml 1 g of phosphotungstic acid hydrate was added, followed by 2 ml glacial ethanoic acid and 100 ml absolute ethanol. The final solution was filtered and stored. Crossmon's trichrome stain was followed by a haematoxylin counter-stain. Using this procedure, the colloid in thyroid follicles stains brightly red, which facilitates digital analysis of images.

Immunocytochemistry

Serial sections were incubated with 2% H₂O₂ and 10% normal goat serum in ice-cold phosphate-buffer to inactivate endogenous peroxidase activity and to block non-specific antigenic sites. Sections were then incubated overnight with a polyclonal antiserum against human thyroxin (MP Biomedicals, Irvine, CA, USA) at a dilution of 1:5,000. Then, sections were incubated for 1 hour with 1:200 biotinylated goat anti-rabbit secondary antibody (VectaStain, Vector Laboratories, Burlingame, CA, USA) and incubated for 30 min with VectaStain ABC reagent. Antibody binding was detected with 0.025% 3,3-diaminobenzidine (Sigma, St. Louis, MO, USA) in the presence of 0.02% H₂O₂.

Morphological data analysis

Sections were analysed with a Leica DM-RBE light microscope (Leica, Wetzlar, Germany). Each thyroid follicle in the section was digitally photographed at 20-times magnification. The colloid in every follicle was manually selected using Adobe Photoshop 7.0 software and quantified using MetaMorph 6 software (Universal Imaging, Downingtown, PA, USA). The epithelial cell height of three thyrocytes per follicles in five follicles per tissue per fish, was digitally determined. The area, perimeter, maximal diameter, length and width of every single colloid cross-section was assessed. Shape was determined with three dimensionless shape descriptors: form factor, roundness and aspect ratio, and were calculated as follows (Ponton, 2006):

$$\text{Form factor} = 4 \cdot \pi \cdot \text{Area} / \text{Perimeter}^2$$

The form factor is the inverse ratio of the squared perimeter of the colloid to the squared perimeter of a circle with the same area, and expresses the evenness of the colloid's outline. The more the form factor approaches 1, the more the outline resembles the outline of a circle.

$$\text{Roundness} = 4 \cdot \text{Area} / \pi \cdot (\text{Maximal Diameter})^2$$

Roundness is calculated as the ratio of the area of the colloid to the area of a circle with the same diameter. The larger the ratio, the better the colloid's shape resembles a circle (roundness = 1).

$$\text{Aspect ratio} = \text{Maximal Length} / \text{Maximal Width}$$

The aspect ratio is calculated by dividing the maximal length of the colloid by the perpendicular maximal width. A larger aspect ratio indicates a more elongated colloid; a ratio of 1 corresponds to a perfect circular colloid.

An initial analysis of frequency distributions revealed that the area, perimeter, form factor, roundness and aspect ratio are not distributed in a Gaussian fashion. We therefore chose the mode as a descriptive statistic. Since a histogram requires a subjective selection of bin width and endpoint, which may greatly affect the shape of the histogram and mode, we have determined the frequency distribution by kernel density estimation (Parzen, 1962) using an add-in utility (version 1.0e) for Microsoft® Excel from the Royal Society of Chemistry (Thompson, 2006).

In-vitro incubations

Total subpharyngeal area, head kidney and kidney were dissected from 14 carp (61 ± 15 g). Tissues were weighed and diced into approximately 1-mm³ sized fragments and immediately transferred to a microtitre plate in 3 ml Hepes-Tris-buffered medium (pH 7.4) saturated with

carbogen (95% O₂-5% CO₂) and allowed to recover for 1 hour. Then, tissues were transferred
195 to a clean plate in 3 ml of the aforementioned buffer and exposed to 10 mIU/ml bovine TSH
(bTSH) (Sigma, St. Louis, MO, USA) or saline vehicle. Tissues were incubated for 24 h at 23°C,
after which the incubation medium was sampled. Total T4 (thyroxine, or 3,5,3'5'-tetraiodo-
thyronine) in the medium was determined using a commercially available enzyme linked
immunoassay (Research Diagnostics, Inc., Flanders, NJ, USA) according to manufacturer's
200 instructions. Thyroxine spiked samples gave representative read-outs.

Thyroid hormone extraction

Several different methods that are based on extraction with Tris-HCl buffer, ethanol, methanol,
butanol, or chloroform, were tested. We found that a combination of Tris-HCl buffer and
205 chloroform to be most efficient in extracting radioactivity. First, tissues were homogenised as
described above. Then, 25 mg of pancreatin (Merck, Darmstadt, Germany), suspended in 0.1 M
Tris-HCl buffer (pH 8.7), was added to 1 ml of subpharyngeal area, head kidney and kidney
homogenates as described by Tong and Chaikoff (1957) which were then incubated for 17 h at
35°C. Chloroform (1.5 ml) was added and the incubate was vigorously mixed for 2 min and
210 centrifuged at 4°C (4000 g, 15 min). The water phase was collected by aspiration and stored at -
20°C until further analysis. One ml of 0.1 M Tris-HCl buffer (pH 8.7) was added to the
remainder of the chloroform/pancreatin mixture, mixed for 10 min, and allowed to incubate for
48 h at 4°C. The mixture was then centrifuged at 4°C (4000 g, 15 min) and the water phase was
aspirated and stored at -20°C until further analysis. The extraction procedure was repeated once,
215 after which the radioactivity of the pooled 3 water phases and of the remaining chloroform/
pancreatin mixture was determined.

Sephadex LH-20 column chromatography

Sephadex LH-20 column chromatography was performed as described by Mol and Visser
220 (1985). In short, glass pipettes were filled with 1 ml Sephadex LH-20 (Amersham Biosciences,
Uppsala, Sweden) suspension in water (10% w/v) and equilibrated with 3 volumes of 1 ml 0.1
M HCl. One-hundred μ l of plasma, bile and extracts of the subpharyngeal area, head kidney and
kidney were deproteinized with four volumes of methanol and centrifuged at 4°C (4000 *g*, 15
min). The supernatants were acidified with one volume of 1 M HCl and loaded on to the
225 column. The samples were then eluted from the column with two volumes of 1 ml 0.1 M HCl
to separate free iodide, 6 volumes of 1 ml H₂O to separate water-soluble conjugated forms of
iodothyronines, and 3 volumes of 1 ml 0.1 M NH₃/EtOH to separate native iodothyronines.
The radioactivity of the collected fractions was measured in a γ -counter.

230 *Statistics*

All data are presented as mean values \pm s.d. Differences between groups were assessed by one-
way ANOVA and Tukey's post hoc test. Statistical significance was accepted at $P < 0.05$ (two-
tailed), probabilities are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

235 **Results**

Autoradiography

Autoradiography demonstrated ¹²⁵I in the subpharyngeal area of tilapia. The radioactivity we
observed in the intestinal tract is most probably caused by drinking (Fig. 1A, B). In carp, ¹²⁵I was
evident in the kidney but not in the subpharyngeal area (Fig. 1C, D). Furthermore, the gall
240 bladder of carp contained radioactivity (Fig. 1E, F) which observation is in contrast with tilapia.
Exposure to perchlorate blocked iodide accumulation in subpharyngeal area of tilapia (Fig. 1G,
H) and carp kidney, although radioactivity was still present in carp gall bladder (Fig. 1I, J).

¹²⁵Iodide tissue distribution

245 We retrieved $97 \pm 14\%$ of the nominal amount of injected radioactivity from the tissue extracts. In tilapia, the subpharyngeal area maximally accumulated ¹²⁵I 31-fold over plasma levels at 96 h into the chase (Table 1). All tissues other than the subpharyngeal area showed a decrease in radioactivity during this period (Fig. 2A). In carp, kidney, head kidney and bile were the only compartments where radioactivity accumulated (Fig. 2B). In carp, kidney tissue was able to
250 accumulate ¹²⁵I more than 500-fold relative to plasma radioactivity at 96 h. The head kidney and subpharyngeal area of carp accumulated iodide as well, 84- and 22-fold, respectively, compared to plasma (Table 1). While carp bile accumulated ¹²⁵I 37-fold at 96 h, tilapia bile radioactivity increased only 2-fold (Table 1).

255 *Histology and morphological analysis*

We could only detect thyroid follicles in the subpharyngeal area of tilapia, and this observation is corroborated by the ¹²⁵I tissue distribution shown in Fig. 2A. In carp, thyroid follicles were observed in kidney, subpharyngeal area and head kidney (Fig. 3A, B, C). Thyroxine-immunoreactivity confirmed that the follicles found in all three tissues are indeed thyroid
260 follicles (Fig. 3D, E, F). On average, 1391 ± 196 ($n = 4$) follicle cross sections were observed in carp, of which $87 \pm 2\%$ is located within the kidney, $3 \pm 1\%$ within the head kidney, and $10 \pm 2\%$ within the subpharyngeal area.

Fig. 4 illustrates the non-Gaussian distribution of the frequency distribution of the area (Fig. 4A), the perimeter (Fig. 4B), the form factor (Fig. 4C), the roundness (Fig. 4D) and the
265 aspect ratio (Fig. 4D) of thyroid follicles' colloids in the subpharyngeal area, head kidney and kidney. The modal value of the area of the kidney colloid is significantly smaller than that of the colloid in the subpharyngeal area and head kidney (Table 2). Also, the mode of the perimeter of the colloid is significantly smaller in the kidney follicles as compared to head kidney follicles (Table 2). Although the mode of the form factor of the colloid did not differ between the

tissues, the mode of the roundness and aspect ratio did; the colloid in the subpharyngeal area is significantly rounder and less elongated than the colloid in the head kidney (Table 2). No differences ($p = 0.192$) were observed between the epithelial cell height of the subpharyngeal area ($6.04 \pm 0.12 \mu\text{m}$, $n = 4$), head kidney ($6.67 \pm 0.68 \mu\text{m}$, $n = 4$) and kidney follicles ($6.54 \pm 0.43 \mu\text{m}$, $n = 4$).

TSH-mediated T4 release in vitro

Exposure to 10 mIU bTSH/ml for 24 h significantly stimulated the release of T4 from carp kidney and head kidney tissue 1.7- and 3.6-fold, respectively (Fig. 5). Tissue from the subpharyngeal area was unresponsive to 10 mIU bTSH/ml.

¹²⁵Iodide pulse chase

Radio-labeled compounds were extracted with very high recoveries from the subpharyngeal area ($92 \pm 2\%$ of total radioactivity), head kidney ($77 \pm 11\%$) and kidney tissue ($92 \pm 5\%$) in tilapia. Similar efficiencies were obtained for carp tissues ($99 \pm 1\%$, $96 \pm 2\%$, and $96 \pm 2\%$, respectively). In previous studies, extractions of radio-labeled thyroid hormones from tissues with ethanol or methanol combined with chloroform usually yielded recoveries, of less than 70% and ranging from 45% to 94% (Crane et al., 2004; Krysin, 1990; Szisch et al., 2005; Tagawa and Hirano, 1987).

Chromatographic analysis of plasma revealed that the decrease in total radioactivity, as observed in Fig 2., can mainly be attributed to a decrease of the tracer injected (¹²⁵I), which occurs at a faster rate in carp than in tilapia; between 2 h and 48 h after injection, ¹²⁵I plasma levels had decreased by $90 \pm 6\%$ in carp, whereas in tilapia, it had decreased by $48 \pm 31\%$ (Fig. 6). Radio-labeled, *i.e.* de-novo synthesised, thyroid hormones appeared in increasing amounts in tilapia plasma during the experimental chase. In carp, however, newly synthesised thyroid

hormones decreased from 2 h onwards. Conjugated forms of thyroid hormones appear in tilapia plasma following the appearance of newly synthesised thyroid hormones.

The chronology of the appearance of labeled thyroid hormone metabolites in the subpharyngeal area, the head kidney and the kidney differed markedly between tilapia and carp. After an initial accumulation, iodide levels remained essentially constant as of 24 h in the subpharyngeal tissue of tilapia, whereas levels of labeled thyroid hormones and conjugates increased after 24 h (Fig. 7A). In kidney (Fig. 7B) and head kidney tissue (Fig. 7C) of tilapia no accumulation of iodide was observed. Small amounts of labeled thyroid hormones and conjugates appear in these tissues at 48 h, which corresponds with the chronology seen in plasma, suggesting that these compounds originate from plasma.

In carp, maximum radioiodide accumulation in the kidney was reached at 48 h, after which the iodide level remained stable at 96 h (Fig. 7B). Thyroid hormones and thyroid hormone conjugates increased from 24 h onwards, reaching maximum levels at 48 h. Also in carp head kidney, newly synthesised thyroid hormones were observed as of 24 h, while thyroid hormone conjugates were essentially absent in this tissue (Fig. 7C). The subpharyngeal area of carp did not accumulate detectable radioiodide and virtually no radio-labeled thyroid hormones and thyroid hormone conjugates were observed (Fig. 7A).

Chromatographic analysis of bile revealed an increase in radioiodide content in carp bile, whereas in tilapia bile, radioiodide levels did not change significantly (Fig. 8A). Thyroid hormones and conjugated forms of thyroid hormone accumulated in bile of both species. The average total volume of the bile of both fish species did not decrease during the experiment, indicating that the gal bladder had not emptied in the intestinal tract during the chase period of the experiment.

In the ambient water of both fish, iodide, thyroid hormones and thyroid hormone conjugates were observed (Fig. 8B). Equal amounts of iodide and thyroid hormones were excreted by both fish. Conjugated thyroid hormones, however, were only excreted by tilapia.

Discussion

We observed remarkable differences in the location of active endocrine thyroid tissue between carp and tilapia. In carp three thyroid follicle populations, both active and inactive, were identified (in subpharyngeal area, head kidney and kidney). Also, differences at the level of the peripheral metabolism of thyroid hormones were observed. Not only did plasma clearance differ, also the route of thyroid hormone excretion varied between carp and tilapia.

In tilapia the subpharyngeal area is the only site to find thyroid follicles, where perchlorate-sensitive iodide accumulation is observed, and where thyroid hormones are synthesised *de novo*. This demonstrates, for tilapia, a location and activity typical for the teleostean thyroid gland. The anatomical location of the thyroid gland in carp, however, deviates from that in tilapia. In carp, the renal tissues display thyroid activity as evidenced by iodine accumulation, confirming the observations in carp by Leray and Febvre (1968) and Lysak (1964). Although both head kidney and kidney in carp synthesise thyroid hormones and secrete thyroid hormones following TSH stimulation, the head kidney can have only a moderate share in total thyroid output. Not only is the kidney the foremost iodide accumulating tissue in carp, which is inhibited by perchlorate, it also harbours the largest amount of thyroid tissue: a mere 87% of the total thyroid follicle population, as opposed to 3% in the head kidney. This suggests a significant role for the kidney thyroid follicles in thyroid economy of carp.

The most striking aspect of this study is the absence of iodine accumulation and thyroid hormone synthesis in the subpharyngeal area of carp, despite the presence of thyroid follicles. Even more so, we show that carp subpharyngeal thyroid follicles do not have the capacity to release thyroid hormones upon stimulation with TSH *in vitro*, whereas renal thyroid follicles do. This, together with the high prevalence (87%), establishes the kidney as the anatomical site of the thyroid gland in this species. These results may pose questions whether to (re-)consider the term “heterotopic” in conjunction with “thyroid follicles” in common carp. In goldfish, a species closely related to the common carp, the subpharyngeal thyroid follicles are active and are

responsible for 11 to 40 % of total iodide accumulation (Chavin and Bouwman, 1965; Peter, 1970), leaving a considerable role for subpharyngeal thyroid follicles in the uptake of iodide in this species. However, as in common carp, the subpharyngeal follicles appear not to be
350 responsive to T4 treatment; changes in thyroid activity, *i.e.* iodine accumulation and epithelial cell height, are primarily mediated through interrenal thyroid follicles, which shows that the thyroid populations are not physiologically equivalent (Peter, 1970). Histologically, the subpharyngeal follicles in carp appear normal, active and similar to kidney thyroid follicles, as evidenced by the epithelial cell height that does not differ significantly from that of kidney
355 follicles. Differences in size and shape were observed between the thyroid follicle populations, *viz.* the colloid of kidney follicles is the smallest, and subpharyngeal follicles appear to be more round than renal follicles. Whether these differences reflect differences in thyroid gland physiology requires further investigation.

Although the subpharyngeal thyroid follicles in carp did not incorporate radioiodide to a
360 significant degree or synthesise thyroid hormones *de novo*, these subpharyngeal follicles do show T4 immunoreactivity. Apparently these follicles do have an intrinsic capacity to synthesise thyroid hormones. Whether the rate of thyroid hormone synthesis is too slow to detect within the 96 h of the experimental chase, or whether these follicles were active during earlier life stages and are now dormant (and still contain T4) remains to be determined. Interestingly,
365 several urodeles are able to complete a full life cycle without metamorphosis, so-called neoteny (Rosenkilde and Ussing, 1996). Because of an impaired thyroid system these amphibians are unable to release a surge of thyroxine, necessary to initiate metamorphosis. Although intact and functional, their thyroid system is relatively inactive at several levels of the thyroid axis, from the central regulation of the thyroid gland to the peripheral deiodination of thyroid hormones.
370 Neoteny is also described in fish; during adult life the ice goby (*Leucopsarion petersii*) still exhibit several larval characteristics, indicative of an impaired metamorphosis. During its development, the thyroid follicles are smaller and have a lower epithelial cell height when compared to a

metamorphic goby species; also no TSH immunoreactivity was observed in the pituitary (Harada et al., 2003). Although carp are not neotenic, further research on the carp subpharyngeal thyroid follicles may provide more insight in the mechanisms controlling the non-pharmacologically induced inactivity of the thyroid gland as observed in neotenic organisms. A possible mechanism could be the temporal expression of active and/or inactive splice variants of key-regulators of thyroid hormone synthesis, e.g. TSH-receptor, sodium-iodide symporter or thyroglobulin.

It is unclear why the functional endocrine thyroid tissue is located in the kidney and not in the subpharyngeal area. We hypothesise that two, potentially functional, thyroid populations with different sensitivities to thyrotropic factors, or with different synthesising properties, confer an accurate regulation of thyroid gland output in response to a demand for systemic thyroid hormone. It can also be hypothesised, regarding the close juxtaposition of the extra-pharyngeal thyroid follicles to specific cell-types in the head kidney and kidney, that thyroid hormones have a paracrine effect on interrenal (cortisol-producing), chromaffin (catecholamine-producing), and/or haematopoietic cells or on nephron structures. Paracrine relationships between the stress axis and immune system have already been demonstrated in the multifunctional carp head kidney (Metz et al., 2006). Attempts to demonstrate a direct *in-vitro* effect of thyroid hormones on the release of cortisol in carp head kidney have not been successful yet, even though thyroxine injection in common carp resulted in decreased levels of plasma cortisol (Geven et al., 2006).

The presence of ^{125}I -radioactivity in carp bile and its absence in tilapia bile already suggest a faster turnover rate of thyroid hormones in carp than in tilapia. This is corroborated by the faster clearance of iodide and thyroid hormones from plasma, and the accumulation of iodide and thyroid hormone conjugates in bile of carp compared to tilapia. Not considering differences between species, these results appear to contradict the general idea that higher

temperatures will result in increased thyroid activity (Eales et al., 1982), as our carp are held at a temperature that is 5°C lower than that of tilapia.

400 The appearance of thyroid hormone conjugates in the plasma of tilapia is consistent with the observations by DiStefano et al. (1998), who observed a significant amount of T3-glucuronides in plasma of Mozambique tilapia after i.p. injection with [¹²⁵I]T3 ([¹²⁵I]3,5,3'-tri-iodothyronine). Although thyroid hormone sulfates are found in sera of several mammals (Santini et al., 1993; Wu et al., 1992; Wu et al., 1993), and indirect evidence exists for thyroid
405 hormone conjugates in plasma of European plaice (*Pleuronectes platessa* L.) (Osborn and Simpson, 1969), the Mozambique tilapia appears to be the only vertebrate in which plasma thyroid hormone glucuronides are observed. By injection of trace amounts of radioiodide instead of radio-labeled thyroid hormones, we circumvented the possibility of altering the thyroid status of the fish. The injection of radioiodide, as opposed to radio-labeled thyroid hormones, also
410 allowed us to speculate on the anatomical site where conjugated thyroid hormones are produced. Whereas thyroid hormone conjugates were observed in tilapia plasma at 48 and 96 h after i.p. injection, they were already present in the subpharyngeal area at 24 h, suggesting that the glandular thyroid gland itself may be responsible for the production of thyroid hormone conjugates in tilapia plasma. In this respect, the finding of conjugated forms of thyroid
415 hormones in the kidney of common carp, which harbours the functional thyroid, is remarkable.

The thyroid hormone conjugates in tilapia plasma have been suggested to function as a pool of thyroid hormones from which, by deconjugation, a rapid mobilization of bioactive thyroid hormones is available (DiStefano et al., 1998). Our results, however, suggest that the thyroid hormone conjugates in tilapia plasma are involved in the excretion of thyroid hormones,
420 through other routes than bile. The appearance of thyroid hormone conjugates in the ambient water coincides with the appearance of thyroid hormone conjugates in the plasma, suggesting that thyroid hormone conjugates are excreted via plasma, possibly through the gill or kidney. Since the volume of the gall bladders did not decrease during the experiment and leakage of

thyroid hormone conjugates over the gal bladder wall in fish is negligible (Collicutt and Eales,
425 1974), the thyroid hormone conjugates in the ambient water are unlikely to originate from the
bile. It is striking that in channel catfish (*Ictalurus punctatus*), 8.1 % of all injected [¹²⁵I]T4 is
excreted via routes other than the gal bladder (Collicutt and Eales, 1974) and that in rainbow
trout (*Oncorhynchus mykiss*), 8.2 % and 6.7 % of injected [¹²⁵I]T4 and [¹²⁵I]T3, respectively, was
excreted via urine (Parry et al., 1994). These reported percentages are of the same order of
430 magnitude as the observed percentage (8.3%) of thyroid hormone conjugates found in tilapia
plasma, supporting the idea that plasma thyroid hormone conjugates in tilapia may function in
the excretion of thyroid hormone metabolites (DiStefano et al., 1998).

In summary, we have shown that thyroid hormone synthesis, anatomical location and activity of
thyroid tissue, and thyroid hormone excretion in teleost fish already differ greatly between two
435 species. The most distinct feature of teleost thyroid physiology observed in this study is the
presence of a completely functional endocrine thyroid gland in the renal tissues of common
carp. This finding may open new possibilities for *in vitro* studies on fish thyroid.

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References

Agrawala, N. and Dixit, R. K. (1979). Seasonal variations in the pharyngeal and
445 pronephric thyroid tissues of the fresh water teleost *Puntius sophore* (Ham). *Z. Mikrosk. Anat.*
Forsch. **93**, 138-146.

Baker, K. F. (1958). Heterotopic thyroid tissues in fishes I. The origin and development
of heterotopic thyroid tissue in platyfish. *J. Morphol.* **103**, 91-129.

- Berg, O., Edgar, M. and Gordon, M.** (1953). Progressive growth stages in the
450 development of spontaneous thyroid tumors in inbred swordtails *Xiphophorus montezumae*. *Cancer Res.* **13**, 1-8.
- Bhattacharya, S., Das, R. H. and Datta, A. G.** (1976). Iodine metabolism in dispersed
pharyngeal and head kidney teleostean thyroid cells obtained by continuous trypsinization. *Gen. Comp. Endocrinol.* **30**, 128-130.
- Blanton, M. L. and Specker, J. L.** (2007). The hypothalamic-pituitary-thyroid (HPT)
455 axis in fish and its role in fish development and reproduction. *Crit. Rev. Toxicol.* **37**, 97-115.
- Blasiola, G. C., Jr, Turnier, J. C. and Hurst, E. E.** (1981). Metastatic thyroid
adenocarcinomas in a captive population of kelp bass, *Paralabrax clathratus*. *J. Natl. Cancer Inst.*
66, 51-59.
- Bongers, A. B. J., Sukkel, M., Gort, G., Komen, J. and Richter, C. J. J.** (1998).
460 Development and use of genetically uniform strains of common carp in experimental animal
research. *Lab. Anim.* **32**, 349-363.
- Chavin, W. and Bouwman, B. N.** (1965). Metabolism iodine and thyroid hormone
synthesis in the goldfish, *Carassius auratus* L. *Gen. Comp. Endocrinol.* **5**, 493-503.
- Chopra, I. J., Chua Teco, G. N., Eisenberg, J. B., Wiersinga, W. M. and Solomon,
465 D. H.** (1982). Structure-activity relationships of inhibition of hepatic monodeiodination of
thyroxine to 3,5,3'-triiodothyronine by thiouracil and related compounds. *Endocrinology* **110**, 163-
168.
- Collicutt, J. M. and Eales, J. G.** (1974). Excretion and enterohepatic cycling of ¹²⁵I-L-
470 thyroxine in channel catfish, *Ictalurus punctatus* Rafinesque. *Gen. Comp. Endocrinol.* **23**, 390-402.
- Crane, H. M., Pickford, D. B., Hutchinson, T. H. and Brown, J. A.** (2004).
Developmental changes of thyroid hormones in the fathead minnow, *Pimephales promelas*. *Gen. Comp. Endocrinol.* **139**, 55-60.

- Crossmon, G.** (1937). A modification of Mallory's connective tissue stain with a
475 discussion of the principles involved. *Anat. Rec.* **69**, 33-38.
- DiStefano, J. J., 3rd, Ron, B., Nguyen, T. T., Weber, G. M. and Grau, E. G.**
(1998). 3,5,3'-Triiodothyronine (T₃) clearance and T₃-glucuronide (T₃G) appearance kinetics in
plasma of freshwater-reared male tilapia, *Oreochromis mossambicus*. *Gen. Comp. Endocrinol.* **111**, 123-
140.
- Eales, J. G. and Brown, S. B.** (1993). Measurement and regulation of thyroidal status
480 in teleost fish. *Rev. Fish Biol. Fish.* **3**, 299-347.
- Eales, J. G., Chang, J. P., van der Kraak, G., Omeljaniuk, R. J. and Uin, L.** (1982).
Effects of temperature on plasma thyroxine and iodide kinetics in rainbow trout, *Salmo gairdneri*.
Gen. Comp. Endocrinol. **47**, 295-307.
- Finnson, K. W. and Eales, J. G.** (1996). Identification of thyroid hormone conjugates
485 produced by isolated hepatocytes and excreted in bile of rainbow trout, *Oncorhynchus mykiss*. *Gen.*
Comp. Endocrinol. **101**, 145-154.
- Fournie, J. W., Wolfe, M. J., Wolf, J. C., Courtney, L. A., Johnson, R. D. and**
Hawkins, W. E. (2005). Diagnostic criteria for proliferative thyroid lesions in bony fishes.
490 *Toxicol. Pathol.* **33**, 540-551.
- Frisén, L. and Frisén, M.** (1967). Analysis of the topographic distribution of thyroid
activity in a teleost fish: *Carassius carassius* L. *Acta Endocrinol.* **56**, 533-546.
- Geven, E. J. W., Verkaar, F., Flik, G. and Klaren, P. H. M.** (2006). Experimental
hyperthyroidism and central mediators of stress axis and thyroid axis activity in common carp
495 (*Cyprinus carpio* L.). *J. Mol. Endocrinol.* **37**, 443-452.
- Gudernatsch, J. F.** (1911). The thyroid of teleosts. *J. Morphol.* **21**, 709-782.
- Harada, Y., Harada, S., Kinoshita, I., Tanaka, M. and Tagawa, M.** (2003). Thyroid
gland development in a neotenic goby (ice goby, *Leucopsarion petersii*) and a common goby
(ukigori, *Gymnogobius urotaenia*) during early life stages. *Zool. Sci.* **20**, 883-888.

- 500 **Krysin, E.** (1990). Comparison of different methods for iodothyronines extraction from animal tissues. *Pol. Arch. Weter.* **30**, 39-48.
- Leatherland, J. F. and Down, N. E.** (2001). Tumours and related lesions of the endocrine system of bony and cartilaginous fishes. *Fish Fisheries* **2**, 59-77.
- 505 **Leray, C. and Febvre, A.** (1968). Influence de l'hypothermie sur la physiologie thyroïdienne d'un poisson marin (*Mugil auratus* R.) et d'un poisson d'eau douce (*Cyprinus carpio* L.). Comparaisons entre thyroïde pharyngienne et thyroïde hétérotypique. *C. R. Seances Soc. Biol. Fil.* **162**, 727-731.
- Lysak, A.** (1964). Thyroid centres in carp and in some other teleost fishes revealed by iodine I¹³¹. *Acta Biol. Crac. Ser. Zool.* **7**, 21-50.
- 510 **Metz, J. R., Huising, M. O., Leon, K., Verburg-van Kemenade, B. M. L. and Flik, G.** (2006). Central and peripheral interleukin-1beta and interleukin-1 receptor I expression and their role in the acute stress response of common carp, *Cyprinus carpio* L. *J. Endocrinol.* **191**, 25-35.
- Mol, J. A. and Visser, T. J.** (1985). Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. *Endocrinology* **117**, 1-7.
- 515 **Nigrelli, R. F.** (1952). Spontaneous neoplasms in fishes. VI. Thyroid tumors in marine fishes. *Zoologica* **37**, 185-198.
- Osborn, R. H. and Simpson, T. H.** (1969). Thyroxine metabolism in plaice (*Pleuronectes platessa* L.). *Gen. Comp. Endocrinol.* **7**, 524.
- 520 **Parry, J. E., Zhang, C. and Eales, J. G.** (1994). Urinary excretion of thyroid hormones in rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **95**, 310-319.
- Parzen, E.** (1962). On estimation of a probability density function and mode. *Ann. Math. Stat.* **33**, 1065-1076.
- Peter, R. E.** (1970). Comparison of the activity of the pronephric thyroid and the pharyngeal thyroid of the goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* **15**, 88-94.

- 525 **Ponton, D.** (2006). Is geometric morphometrics efficient for comparing otolith shape
of different fish species? *J. Morphol.* **267**, 750-757.
- Qureshi, T. A.** (1975). Heterotopic thyroid follicles in the accessory mesonephric lobes
of *Heteropneustes fossilis* (Bloch). *Acta Anat.* **93**, 506-511.
- Qureshi, T. A., Belsare, D. K. and Sultan, R.** (1978). Head-kidney thyroid in some
530 Indian teleosts. *Z. Mikrosk. Anat. Forsch.* **92**, 352-358.
- Qureshi, T. A. and Sultan, R.** (1976). Thyroid follicles in the head-kidney of teleosts.
Anat. Anz. **139**, 332-336.
- Rijntjes, N. V. M., van de Putte, L. B. A., van der Pol, M. and Guelen, P. J. M.**
(1979). Cryosectioning of undecalcified tissues for immunofluorescence. *J. Immunol. Methods* **30**,
535 263-268.
- Rosenkilde, P. and Ussing, A. P.** (1996). What mechanisms control neoteny and
regulate induced metamorphosis in urodeles? *Int. J. Dev. Biol.* **40**, 665-673.
- Santini, F., Hurd, R. E., Lee, B. and Chopra, I. J.** (1993). Thyromimetic effects of
3,5,3'-triiodothyronine sulfate in hypothyroid rats. *Endocrinology* **133**, 105-110.
- 540 **Sathyanesan, A. G.** (1963). Functional renal thyroid follicles in wild specimens of the
freshwater teleost *Barbus stigma* (Cuv. et Val.). *Z. Zellforsch. Mikrosk. Anat.* **59**, 530-534.
- Srivastava, S. S. and Sathyanesan, A. G.** (1971). Studies on the histophysiology of the
pharyngeal and heterotopic renal thyroid in the freshwater teleost *Puntius sophore* (Ham.). *Z.*
Mikrosk. Anat. Forsch. **83**, 145-165.
- 545 **Szisch, V., Papandroulakis, N., Fanouraki, E. and Pavlidis, M.** (2005). Ontogeny
of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. *Gen. Comp.*
Endocrinol. **142**, 186-192.
- Tagawa, M. and Hirano, T.** (1987). Presence of thyroxine in eggs and changes in its
content during early development of chum salmon, *Oncorhynchus keta*. *Gen. Comp. Endocrinol.* **68**,
550 129-135.

Thompson, M. (2006). Representing data distributions with kernel density estimates.
AMC Tech. Brief **4**, 1-2.

Tong, W. and Chaikoff, I. L. (1957). Hydrolysis of I^{131} -thyroprotein by pancreatic enzymes. *J. Biol. Chem.* **232**, 939-950.

555 **Wu, S. Y., Huang, W. S., Polk, D., Florsheim, W. H., Green, W. L. and Fisher, D. A.** (1992). Identification of thyroxine-sulfate (T_4S) in human serum and amniotic fluid by a novel T_4S radioimmunoassay. *Thyroid* **2**, 101-105.

Wu, S. Y., Polk, D. H., Huang, W. S., Reviczky, A., Wang, K. and Fisher, D. A.
(1993). Sulfate conjugates of iodothyronines in developing sheep: effect of fetal hypothyroidism.
560 *Am. J. Physiol.* **265**, E115-120.

565

Table 1. Fold-increase in ^{125}I -radioactivity (Bq/g tissue) relative to plasma ^{125}I -radioactivity (Bq/g plasma) at 96 h after i.p.-injection of radioiodide ($n = 6$).

570

	Tilapia	Carp
Subpharyngeal area	31 ± 27	20 ± 13
Head kidney	0.5 ± 0.07	70 ± 62
Kidney	0.6 ± 0.05	544 ± 490
Bile	2 ± 3	37 ± 16

Table 2. Mode of size and morphology descriptors for carp thyroid follicle colloid ($N = 4$). One-way ANOVA was used for statistical evaluation. Different letters indicate significant differences within the column, $P < 0.05$.

	Area	Perimeter			
	(μm^2)	(μm)	Form Factor	Roundness	Aspect Ratio
Subpharyngeal area	1403 ± 226^a	$151 \pm 13^{a,b}$	0.80 ± 0.06^a	0.75 ± 0.01^a	1.18 ± 0.01^a
Head kidney	1446 ± 283^a	166 ± 30^a	0.83 ± 0.05^a	0.70 ± 0.02^b	1.27 ± 0.03^b
Kidney	963 ± 91^b	125 ± 8^b	0.88 ± 0.01^a	$0.72 \pm 0.02^{a,b}$	$1.23 \pm 0.04^{a,b}$

575

Figure legends

Figure 1. Representative autoradiographs of 30 μm , whole body cryosections of tilapia (A, B) and carp (C, D, E, F) exposed to ^{125}I and of tilapia (G, H) and carp (I, J) exposed to ^{125}I and KClO_4 . Dashed circles indicate the position of the subpharyngeal area (1), the gal bladder (2) and the kidney (3). Scale bars indicate 1 cm.

Figure 2. Tissue distribution of the radioactivity, % of the total dosis remaining in the fish at the time of sampling, per gram tissue at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (A, $n = 6$) and carp (B, $n = 6$). One way ANOVA was used for statistical evaluation. Asterisks (*) indicate a significant difference compared to the radioactivity level at 2 h.

Figure 3. Crossmon's staining of 7 μm thick sections of carp subpharyngeal area (A), head kidney (B) and kidney (C). Thyroxine immunohistochemistry on serial sections of carp subpharyngeal area (D), head kidney (E) and kidney (F). Scale bars indicate 200 μm .

Figure 4. Frequency distributions of the area (A), perimeter (B), form factor (C), roundness (D) and aspect ratio (D) of the colloid of all individual thyroid follicles in a representative subpharyngeal area, head kidney and kidney of carp. Insert graphs represent the boxed area, to highlight the mode of the frequency distributions.

Figure 5. TSH (10 mIU/ml) mediated T4 release by carp subpharyngeal area (A, $n = 7$), head kidney (B, $n = 7$) and kidney (C, $n = 7$). One-way ANOVA was used for statistical evaluation.

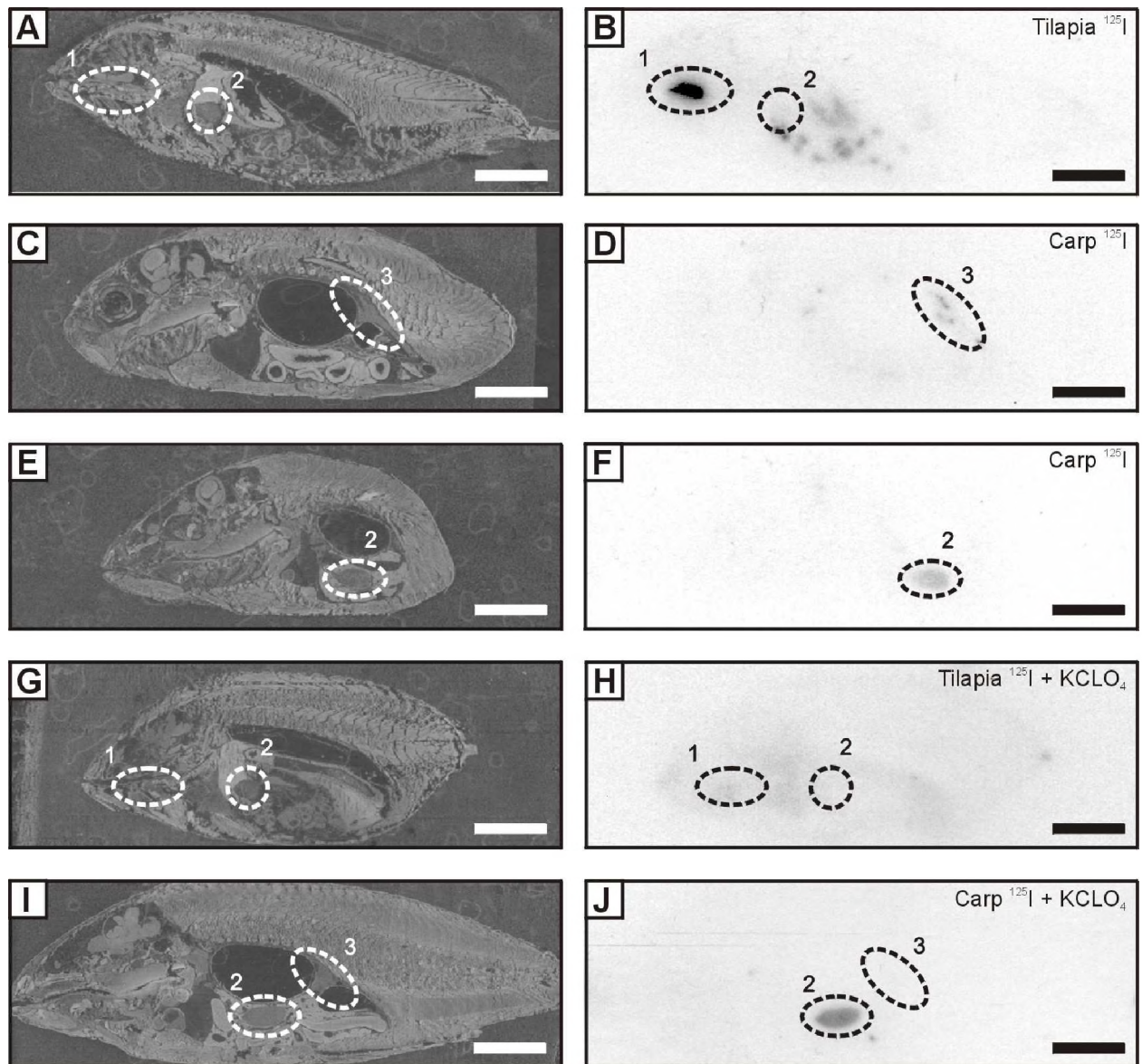
Figure 6. Radioactivity of iodide, TH and TH conjugates fractions, % of the total dosis remaining in the fish at the time of sampling, per gram plasma after Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (closed symbol, \bullet , $n = 6$)

and carp (open symbol, ○, $n = 6$). One-way ANOVA was used for statistical evaluation. To indicate significant different levels of radioactivity compared to the radioactivity level at 2 h, asterisks (*) and plus signs (+) are used for tilapia and carp, respectively.

Figure 7. Radioactivity of iodide, TH and TH conjugates fractions, % of the total dosis remaining in the fish at the time of sampling, per gram tissue, in the subpharyngeal area (A), kidney (B) and head kidney (C), after Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (closed symbol, ●, $n = 6$) and carp (open symbol, ○, $n = 6$). One-way ANOVA was used for statistical evaluation. To indicate significant different levels of radioactivity compared to the radioactivity level at 2 h, asterisks (*) and plus signs (+) are used for tilapia and carp, respectively.

Figure 8. Radioactivity of iodide, TH and TH conjugates fractions, % of the total dosis remaining in the fish at the time of sampling, per gram tissue, in the bile (A) and the ambient water (B), after Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (closed symbol, ●, $n = 6$) and carp (open symbol, ○, $n = 6$). One-way ANOVA was used for statistical evaluation. To indicate significant different levels of radioactivity compared to the radioactivity level at 2 h, asterisks (*) and plus signs (+) are used for tilapia and carp, respectively.

Figures



625 Figure 1.

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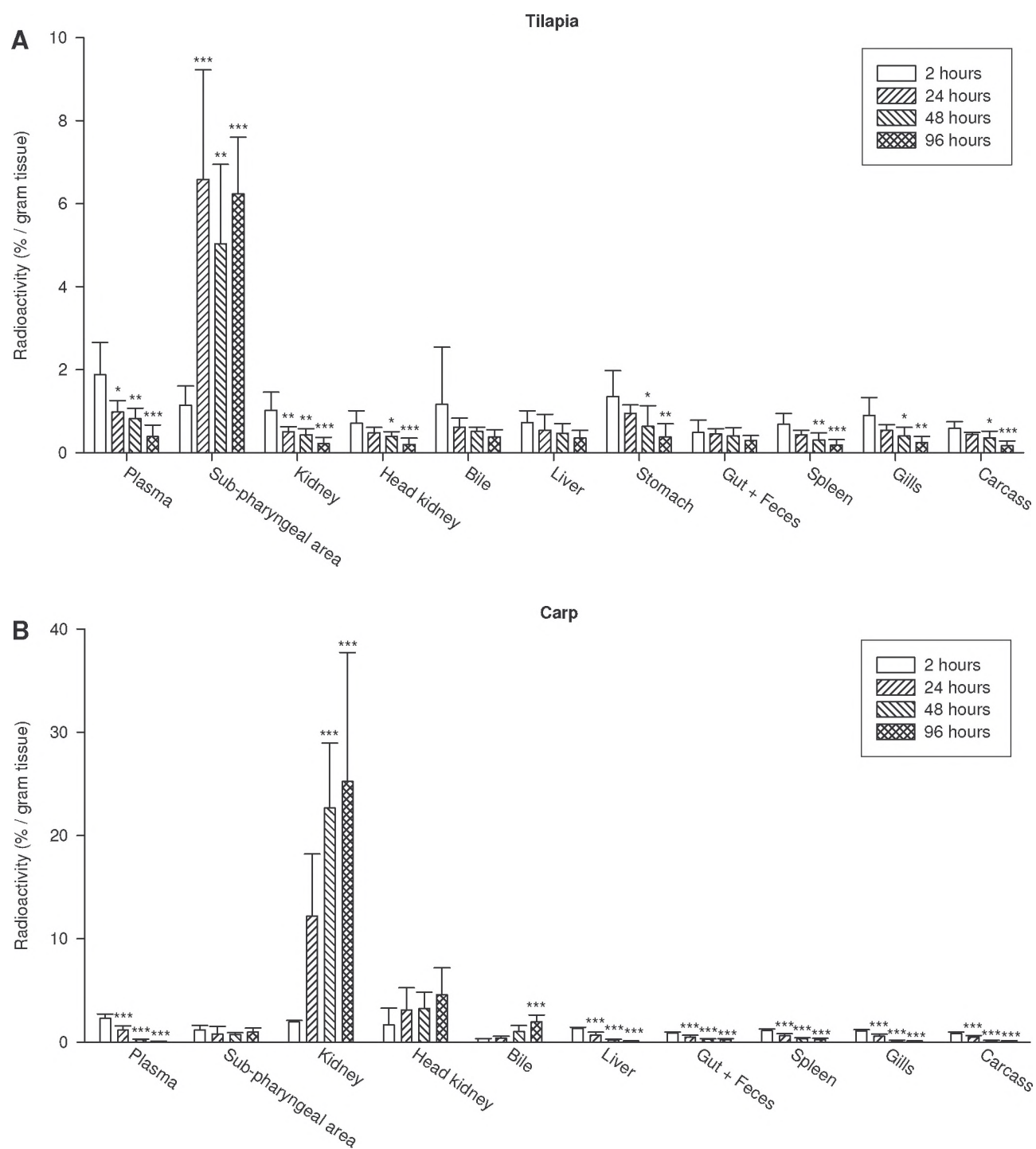


Figure 2.

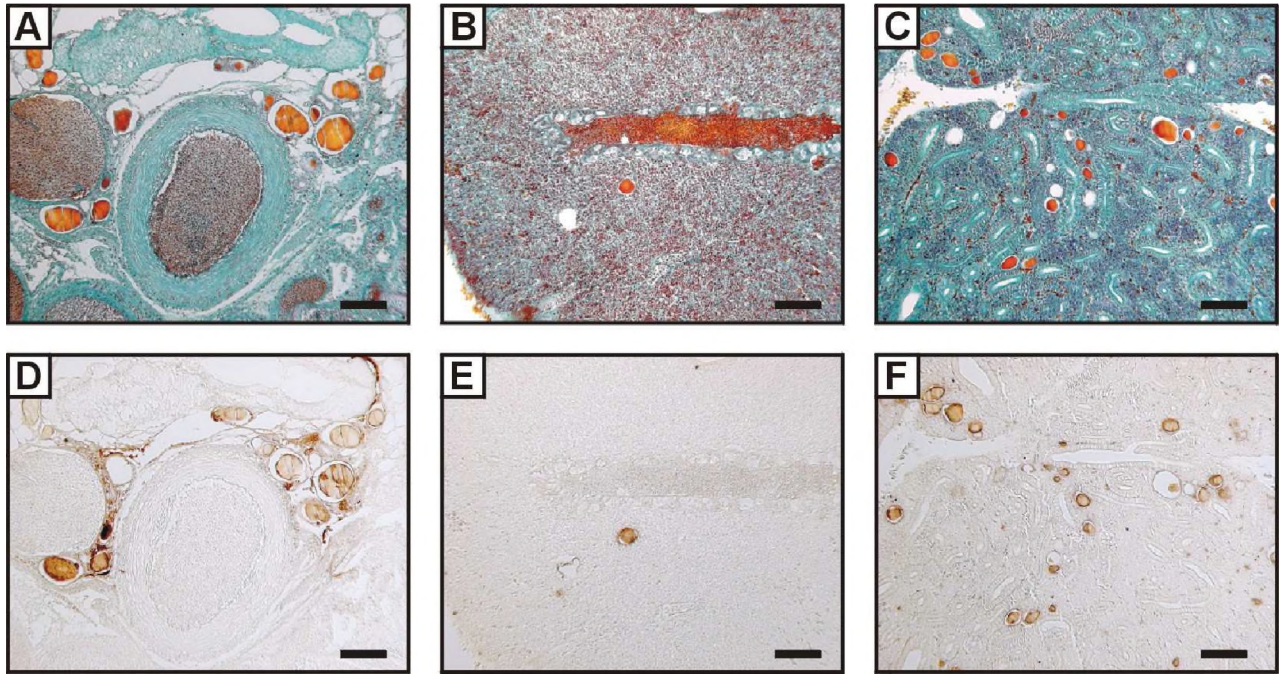


Figure 3.

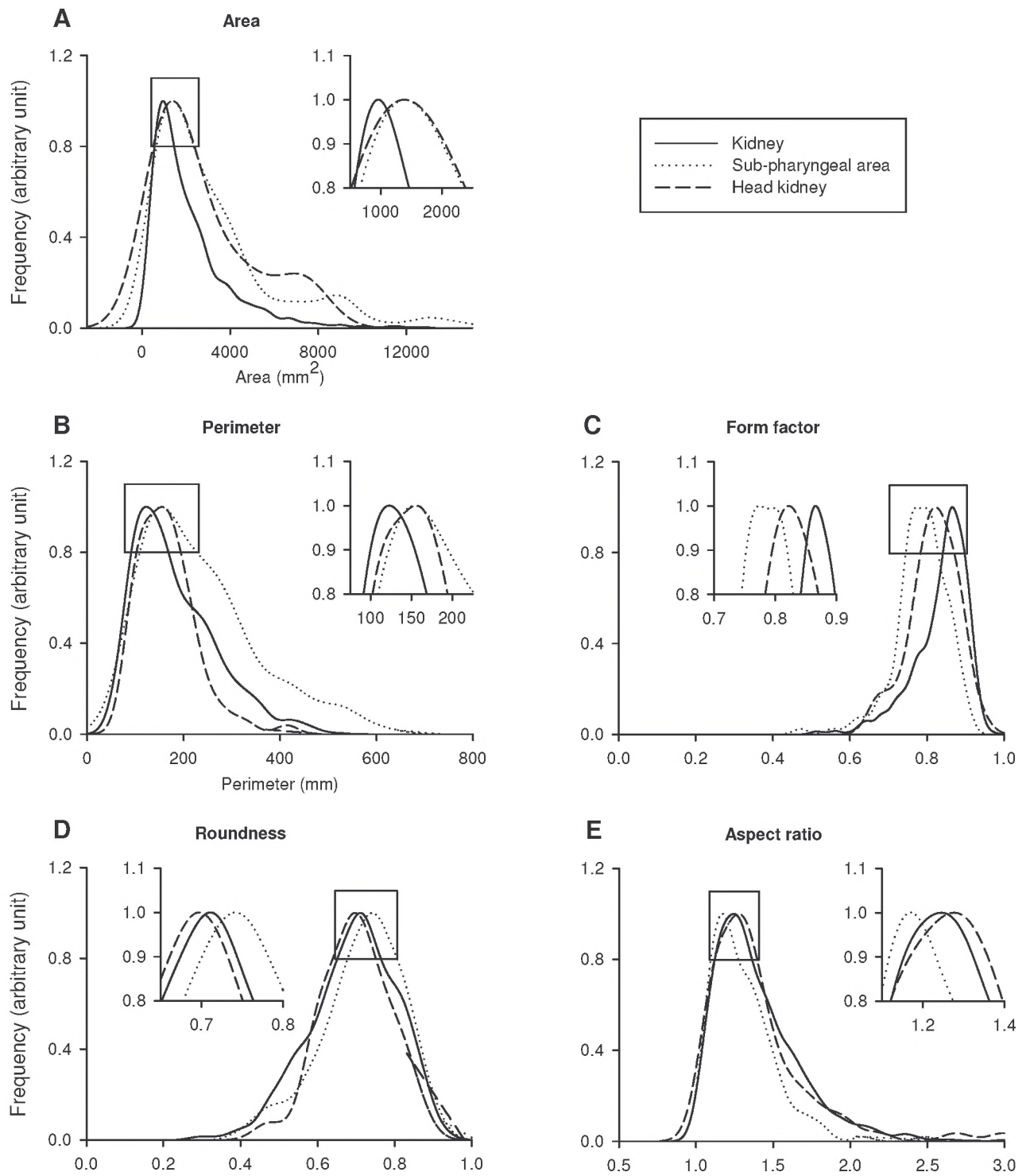


Figure 4.

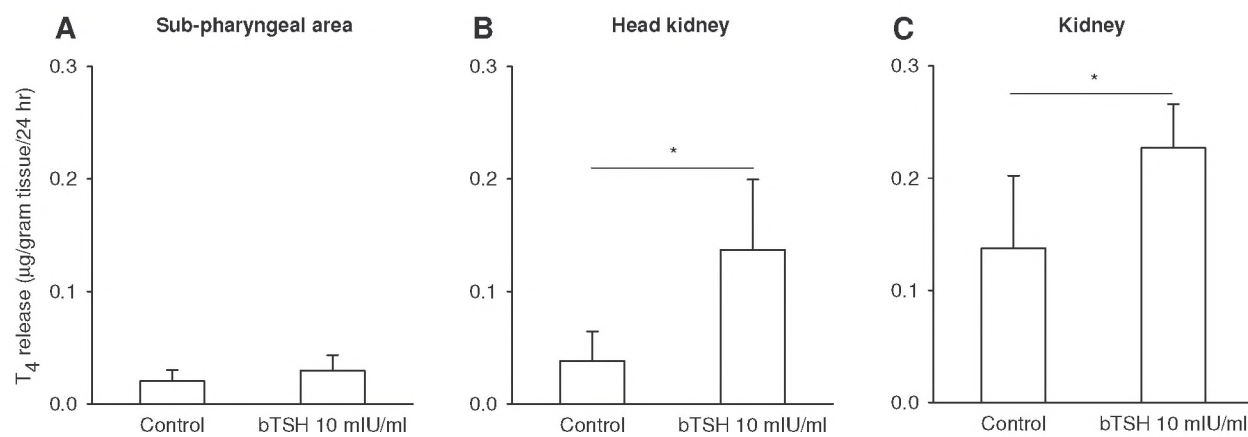
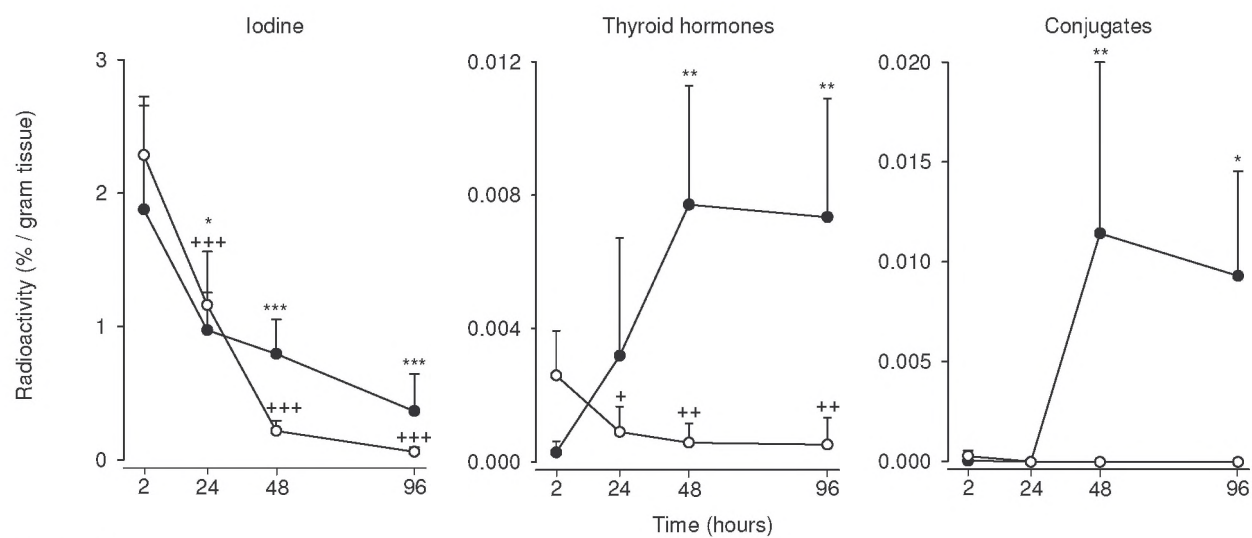


Figure 5.



650 Figure 6.

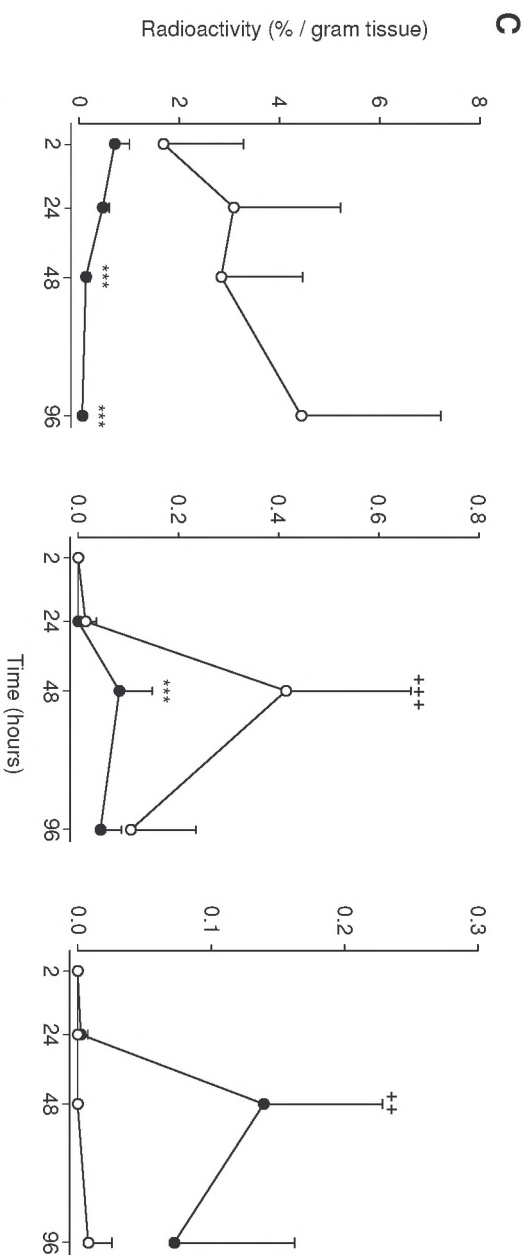
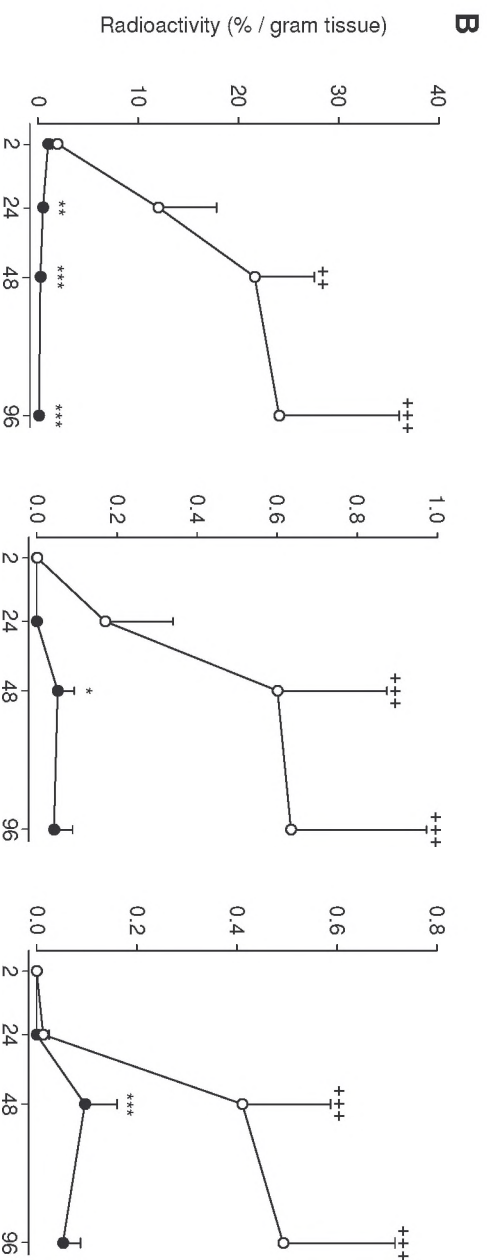
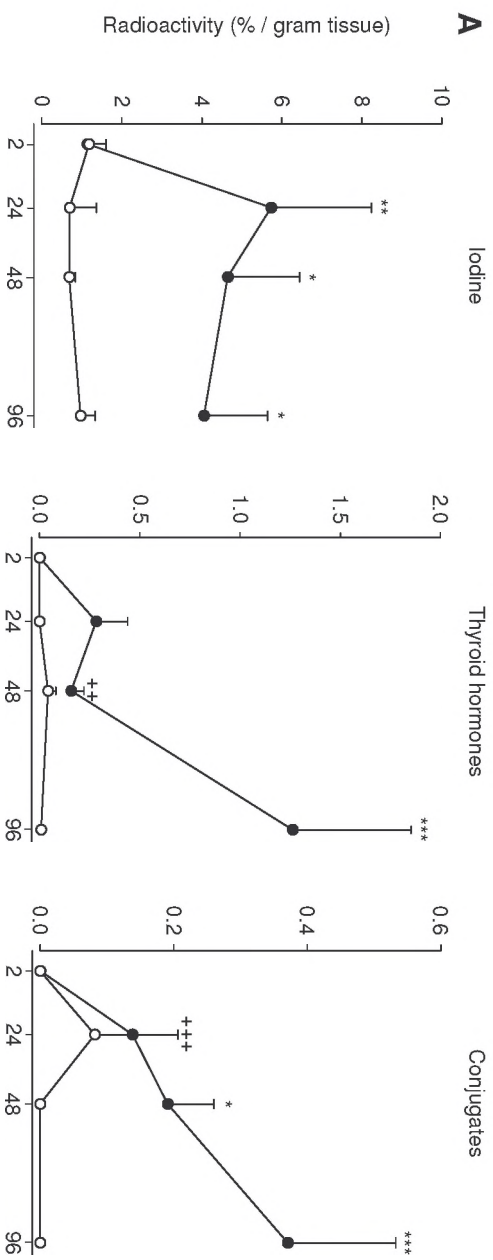
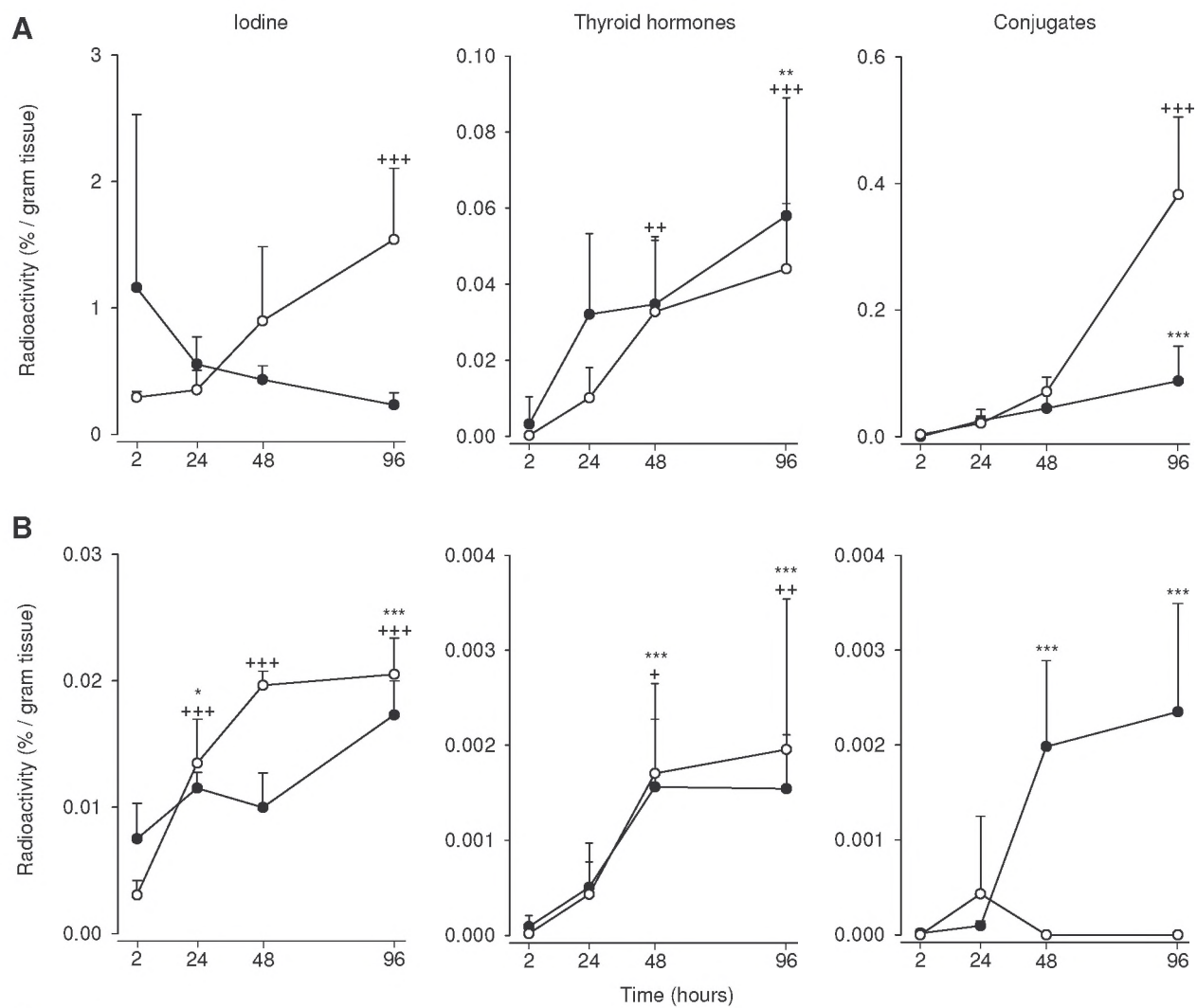


Figure 7.





655 Figure 8.